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Identification, description, and activity of proteins in the tergal glands of the German cockroach, Blattella germanica (L.)

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IDENTIFICATION, DESCRIPTION, AND ACTIVITY OF PROTEINS IN THE TERGAL GLANDS OF THE GERMAN COCKROACH, *BLATTELLA GERMANICA* (L.)

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ABSTRACT

Myers, Aaron J. M.S., Purdue University, May 2015. Identification, description, and activity of proteins in the tergal glands of the German cockroach, *Blattella germanica* (L.). Major Professors: Gary Bennett and Mike Scharf.

German cockroaches are important urban pests that have been linked to asthma and serious allergic reactions in sensitized individuals. In this research project I, (i) identified different proteins expressed in the tergal glands of male German cockroaches, (ii) determined the expression levels of these proteins in different cockroach life stages and tissues, and (iii) investigated the role of the tergal gland alpha-amylase (BGTG-1) protein.

Four major tergal gland proteins were separated on denaturing polyacrylamide gels. With peptide sequencing two of these proteins were identified as alpha-amylase (BGTG-1) and *Blattella germanica* allergen 2 (Bla g 2). Both of these proteins showed highest relative expression in adult male tergal glands as compared to other body parts of adult males, non-gravid females and gravid females. Enzyme assays and RNA interference (RNAi) based silencing of the BGTG-1 gene/protein, provided preliminary evidence that it hydrolyzes starch to release maltose.

Overall, proteins found in the tergal glands of male German cockroaches appear to be serving potentially important roles within the glands. Future research will identify the functional roles of these proteins in cockroach mating behavior and allergen biology.

The ultimate goal of tergal gland biology research is to find ways to disrupt cockroach mating behavior and allergen production and use this knowledge for cockroach management and allergen mitigation.

CHAPTER 1. BIOLOGY OF GERMAN COCKROACHES AND THEIR IMPORTANCE AS URBAN INSECTS

1.1 Introduction

The German cockroach belongs to the family Blattellidae(also known as the wood cockroach family), which includes several well-known members that are considered pest species, including the German cockroach, *Blattella germanica*, and the brown-banded cockroach, *Supella longipalpa* (Roth 1970). The German cockroach is an important urban pest species. It invades homes, restaurants, hospitals, and other places where humans dwell. Cockroaches generally seek food, water and harborage in human dwellings. German cockroaches are known to harbor and transport bacteria on their cuticle, which is especially a problem in the food and healthcare industries (Miller and Meek 2004). The main issue with these cockroaches is their ability to produce airborne allergens which can be inhaled by residents living in cockroach infested places. Cockroach allergens have been associated with asthma and other health risks in humans. Children and the elderly are especially at risk with respect to cockroach allergens (Nojima et al. 2005).

German cockroaches are a pest species that are most commonly found in cracks and crevices in human dwellings; they require food, water and harborage to sustain their populations in urban settings. German cockroaches tend to be mostly nocturnal coming out of harborage mostly at night unless populations are very large in which case cockroaches can be seen moving around throughout the day. German cockroaches hiding

in cracks and crevices, tend to be found in aggregations of mixed aged nymphs and adults (Dambach and Goehlen 1999, Sakuma and Fukami 1993). This is probably due to a few factors, one being water and heat conservation and another being thigmotaxis, where the cockroaches seek out shelters and aggregations where they are receiving touch stimulus on multiple sides of their bodies (Dambach and Goehlen 1999, Sakuma and Fukami 1993). Water loss due to evaporation is reduced when cockroaches aggregate, which allows them to conserve more water and reduce their risk of desiccation, which is especially important for the smaller nymphs (Dambach and Goehlen 1999).

Due to their nocturnal nature and their living habits, German cockroaches create a special problem for pest management professionals (PMPs). Multiple chemical and non-chemical approaches are needed in order to manage German cockroach populations. With German cockroaches, PMPs would use a multifaceted program that uses various tactics and not just insecticides. Integrated Pest Management (IPM) programs utilize and focus on three different tactics: prevention, monitoring, and using low toxicity control agents (Miller and Meek 2004). Prevention of German cockroaches involves altering the physical environment to make it unfavorable for them to thrive and reproduce, which includes sanitation and elimination of food, water and harborage resources. Monitoring of German cockroaches involves using various traps, including sticky traps to determine (i) population size, (ii) population hotspots (i.e., infestation locations), and (iii) efficacy of a particular management plan after treatments have been done (Miller and Meek 2004). When using low toxicity control agents, it’s important to protect non-target organisms (pets, children, etc.) that may come into contact with the insecticide (Miller and Meek 2004). Dust or liquid formulations of insecticides are generally applied to cockroach

harborage areas. Bait formulations of insecticides are applied in the same manner. Bait formulations used for cockroach control consists of a food stimulant, usually a sugar, and high concentration of non-repellent insecticides (Wang and Bennett 2006a). However, with continuous use of insecticides, there is always a risk of the pest insect developing resistance and the German cockroach is no exception.

German cockroaches have developed resistance to various chemicals and baits, including pyrethroids and fipronil (Gondhalekar and Scharf 2012, Wei et al. 2001, Wu et al. 1998). In German cockroaches that are resistant to fipronil, which is a component of some gel bait formulations, research has shown that the resistant individuals have enhanced cytochrome P450-mediated metabolism and a mutation in a target site gene (*resistance to dieldrin; Rdl*) which causes target site insensitivity in the German cockroach nervous system (Gondhalekar and Scharf 2012). Resistance development in a population is due to repeated exposures to a particular insecticide or control measure, which puts a great selection pressure on the population to adapt and change or go extinct. Over time, after repeated exposures to the same or similar insecticides, resistance to a particular insecticide can occur because the individuals that do survive the control treatments are better fit to survive these treatments and they pass their resistance-associated genes to the next generation. Over time with continued insecticide selection pressure the entire population develops the ability to survive control measures that have been repeated many times over each new generation. In German cockroaches, three types of resistance mechanisms have been reported, these are: behavioral, physiological, and metabolic resistance (Wu et al. 1998). Some field populations of German cockroaches have developed behavioral resistance to gel bait formulations. This type of behavioral

resistance is also known as bait aversion resistance. Cockroaches displaying bait aversion avoid feeding on gel baits, when they detect particular sugars that are part of a gel bait formulation (Wang et al. 2006b). Resistance occurs relatively rapidly in most insect populations, such as German cockroaches, due to their prolific rate of reproduction.

1.2 German Cockroach Mating Behavior

German cockroach males and females exhibit intricate mating behaviors. Mating is a multifaceted, complex behavior that requires multiple steps by both sexes. There are multiple chemicals and pheromones at work during this precopulatory process. Both males and females utilize different pheromones to attract conspecifics and these pheromones are released from different parts of their bodies and at different points during the copulatory behavior. The female German cockroach is the one who initiates mating with the release of a long distance sex pheromone, termed “blattellaquinone” (Nojima et al. 2005). When attracting male cockroaches, females assume a “calling” posture, where the female raises her hind legs pushing the thorax and head closer to the standing substrate and raising the abdomen off the substrate while also extending the abdomen. In this “calling” position pheromone producing glands found on the pygidium (the last abdominal segment) release the long distance pheromone into the air away from the substrate (Liang and Schal 1993, Nojima et al. 2005). Releasing the pheromone away from the substrate increases the chances of pheromone being picked up by adult males.

The sex pheromone released by the female is detected by receptors present on antennae of adult male German cockroaches. This elicits courtship and searching behavior in the males (Nojima et al. 2005). Once the male finds a female, there are a series of courtship behaviors exhibited by both sexes. Upon meeting, male and female German cockroaches “antennate” each other, where the male picks up a contact sex pheromone found on the cuticular surface of the female that elicits a specific courtship behavior by the male (Eliyahu et al. 2008). After antenation with a female German cockroach, the male German cockroach rotates his body 180^{o}, lifts his wings to expose

tergal glands present on 7th and 8th abdominal surface to the female (Nojima et al 1999b, Saltzmann et al 2006b). These glands release a pheromonal phagostimulatory secretion that attracts females and other nearby cockroaches to feed on the secretion. When a female feeds on this secretion, it places her in the precopulatory position necessary for mating (Nojima et al. 1999a).

While a female is feeding upon the tergal gland secretion, the male German cockroach pushes his abdomen underneath the female and attempts to connect to the female genitalia using his left phallomere, which is a specialized apparatus that is used as a clasp by the males during the mating process. In the final step of the pre-copulatory process, the male and female turn end to end and begin mating (Nojima et al. 2002).

Many species of cockroaches utilize a similar behavioral pattern for mating. In the cockroach species *Leucophaea maderae,* the male emits a long distance sex pheromone to attract conspecifics, while still employing a similar behavioral pattern as that of the male German cockroach, where upon meeting and anttenating with the female, the male turns and raises his wings to expose the tergal glands, allowing her to feed upon the secretion. He then dips his abdomen underneath the female to attempt copulation (Cornette et al. 2003).

1.3 Tergal Gland Physiology

The German cockroach tergal glands are highly specialized regions of the abdomen found on the 7th and 8th abdominal tergites. Tergal glands are composed of several types of modified epithelial cells, epidermal cells and associated cells that contain microtubules and class III gland cells (Saltzmann et al. 2006a). These different cells are all responsible for transporting the gland secretion to the epidermal surface. The tergal gland secretion is composed of lipids, proteins, oligosaccharides, and volatile chemicals that act as a stimulant to attract conspecifics for mating (Saltzmann et al. 2006b). Several oligosaccharides and trisaccharides have been described and blends of these serve as feeding stimulants for the female cockroach (Nojima et al. 2002). The tergal secretion itself is not considered sex specific, as other adult males and nymphs readily feeding upon this secretion. As such, the tergal secretion is also considered as a regular dietary stimulant (Nojima et al. 1999b). Carbohydrates and lipids present in the tergal secretion act as nutritional feeding stimulants in the German cockroach. More specifically, maltose, a simple sugar found in the tergal gland secretion, elicits strong feeding responses from German cockroach males, females and nymphs (Nojima et al 1999b). Along with the sugars, multiple proteins are found in the tergal gland secretion.

Four main proteins have been reported from the cockroach tergal gland secretion. These proteins are easily separated on SDS-PAGE gels based on their molecular weight. Of these four proteins, only one has been identified and is described as an alpha-amylase enzyme. The proposed function of this protein is to hydrolyze complex carbohydrates to release simple sugars such as maltose (Saltzmann et al 2006). The proposed function of the German cockroach alpha-amylase correlates with the function of a similar protein

found in the tergal glands of another cockroach species, *Leucophaea maderae*. In *L. maderae*, a 72kDa protein has been identified to be within the same family of proteins, the glycosidases, as the alpha-amylase protein found in the German cockroach tergal glands (Cornette et al 2003, Saltzmann et al 2006b).

1.4 Objectives

For my Master's research project, there were 2 main objectives: First, I wanted to determine and identify proteins in the tergal gland secretion of male German cockroaches. With this information, I would then determine the relative expression of transcripts of proteins I am interested in and determine in which body parts of adult males, females and gravid females these transcripts of interest are showing the highest expression levels. My second objective for this project was to look further into the BGTG-1 protein and determine its function in the tergal gland secretion using enzyme assays and RNAi.

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CHAPTER 2. SEPARATION OF PROTEINS FOUND IN GERMAN COCKROACH TERGAL GLANDS AND COMPARATIVE RELATIVE EXPRESSION OF THE PROTEIN-CODING GENES *BGTG-1* AND *BLA G 2*

2.1 Abstract

German cockroach males possess a pair of tergal glands on the 7th and 8th abdominal tergites. These glands contain a secretion that contains a multitude of components, including sugars and proteins. In the studies reported here, we separated and isolated 4 major proteins with approximate molecular weights of 23, 45, 63, and 94kDa. Of these, the 63 and the 45kDa proteins were identified as an alpha-amylase (BGTG-1) and *Blattella germanica* allergen 2 (Bla g 2), respectively. Next, relative expression of the mRNA transcripts of these proteins was determined using quantitative PCR (qPCR) experiments. Either whole insects or individual body parts of males, females, and gravid females were used for qPCR expression analysis. Significant difference in expression were not detected in whole-body samples, but when individual body parts were tested, the tergal glands tended to show the highest expression for both BGTG-1 and Bla g 2 genes. A similar trend was not only seen when comparing the tergal glands to other male body parts, but also to different body parts of non-gravid and gravid females, BGTG-1 is a tergal gland specific protein so higher expression of this enzyme in the tergal gland was expected, but the finding that Bla g 2 is also highly expressed in the tergal glands was surprising and has practical implications.

2.2 Introduction

In the urban environment, there are numerous insect species that are considered pests, either due to aesthetic or medical reasons. An important urban pest is the German cockroach, *Blattella germanica*. Not only is the German cockroach considered an unwanted, aesthetic pest of homes, it is also considered a medical pest because cockroaches produce allergens that become airborne and can be inhaled by human occupants living in infested settings. These airborne allergens can cause asthma and other health problems, especially with children and the elderly (Miller and Meek 2004). Multiple protein allergens, named Bla g 1 through Bla g 8, have been linked to causing asthma, allergic reactions, and eczema in residents that live with German cockroach infestations (Sohn and Kim 2012). There have been numerous recent and ongoing studies to understand varying aspects of German cockroach biology and novel ways to control German cockroach populations.

Recent studies have also been targeted towards understanding tergal gland morphology, its roles in German cockroach behavior, and the functional aspects of the secretion within the tergal glands. German cockroach tergal glands secrete a phagostimulatory solution that lures adult females to feed upon the solution, which places the female into a necessary precopulatory position for mating (Roth 1969). The tergal secretion is composed of various oligosaccharides, proteins, lipids and volatile chemicals and is very attractive to the female for consumption (Saltzmann et al. 2006, Nojima et al. 2002, Sreng and Quennedey 1976, Brossut et al. 1975). The tergal glands are located underneath the 7^{th} and 8^{th} abdominal tergites of the adult male German cockroach (Saltzmann et al. 2006). The tergal gland secretions of males could potentially serve as a

nuptial gift to the female, and it is hypothesized that the female may gain nutritional benefits upon consumption of the tergal secretion (Mondet et al. 2008).

The complete carbohydrate and chemical composition of the tergal secretion has been identified (Nojima et al. 2002). However, identity of only one protein (BGTG-1) has been revealed thus far (Saltzmann et al. 2006). Identification of additional protein/enzyme components present in the tergal gland secretion can further elucidate the gland's functional role in German cockroach mating behavior and reveal potential exploitable aspects of biology for achieving better cockroach control. My objectives in the studies presented here were to (1) use proteomics approaches to identify new tergal gland proteins, (2) determine gene sequences for these proteins using existing German cockroach DNA sequence databases, and (3) determine expression profiles of these genes in different sexes, life stages and tissues.

2.3 Materials and Methods

Insects:

Cockroaches from laboratory colonies of the insecticide-susceptible Johnson Wax (JWax) strain maintained at the Purdue University Entomology Department were used for all experiments. Colonies of the JWax strain were held in plastic boxes and given rodent diet (Harlan-Teklad #8604; Madison, WI) and water *ad libitum*. Rearing boxes were kept at 26^{o}C, 60% relative humidity (RH), and a photoperiod of 12:12 (light:dark). Fifth (last) instar nymphs were isolated and observed every day to record emergence into the adult form. Newly molted adult male and female cockroaches were isolated and aged five to seven days after the imaginal molt because these insects (adult males) have been shown to have maximum expression of tergal gland proteins 5 to7 days after the imaginal molt (Saltzmann et al. 2006). Non-gravid females aged for 5–7 days, gravid females and egg cases were also used in gene expression experiments.

Isolation of tergal gland proteins: Methods used to isolate tergal proteins from male *B. germanica* are outlined by Saltzmann et al. (2006). However, these methods are also described here in brief. Fifth instar nymphs were separated from the main colony and observed daily to record adult emergence time. Newly molted adult male cockroaches were isolated and allowed to age for five to seven days after the imaginal molt because 1 week old adult males have been shown to have maximum expression of tergal gland proteins (Saltzmann et al. 2006).

Cockroaches were anaesthetized using carbon dioxide (CO_2) before collecting samples. Tergal gland proteins were collected using sterile filter paper points made using a punching machine originally designed to make insect mounting points. Filter paper

points were held in forceps and inserted into the tergal gland reservoirs and the contents wicked out. One filter paper point was used for wicking tergal secretion from all four reservoirs of each insect. After wicking the filter paper point was placed into a 1.5mL Eppendorf tube containing 250µl of phosphate-buffered saline solution (PBS; pH 7.6). One replicate consisted of tergal secretion collected from ten insects, and thus, ten filter paper points were placed in 250µl of PBS. Filter paper tips were removed from PBS after ca. 30 minutes. Body surface proteins were collected from 5–7 day old adult males and females served as controls. To collect body surface proteins, filter paper points moistened with PBS were wiped along the tergites and sternites of males or females and then placed into PBS for 30 minutes. Additionally, filter paper points dipped into PBS were also used as experimental controls.

After collection, protein samples were precipitated using cold acetone (-20^{o}C). In brief, 1 ml cold acetone was added to protein or control samples in PBS and incubated overnight in a -20^{o}C freezer. After incubation, proteins were precipitated by centrifugation at 15000g for 15 min at 4^{o}C. Following centrifugation, the supernatant was discarded and the resulting protein pellet was washed twice with 100% ice cold acetone and centrifuged at 7000g for 3 to 5 minutes. The samples were then allowed to dry and rehydrated using 20µl PBS. Protein concentrations were determined on a Nanodrop Spectrophotometer (Thermo Scientific; Wilmington, DE) using a commercial Bradford assay.

Protein separation using gel electrophoresis: Proteins were separated by SDS-PAGE on 10% polyacrylamide gels using the Bio-Rad Criterion system (Hercules, CA). Equal amounts of protein (10µg) were loaded into each lane for each of the gel. Tris-glycine

buffer (pH 8.3) with SDS was used as electrode buffer. Proteins were separated at 200V until the tracking dye (Laemmli Sample Buffer) ran off the gel. A protein ladder (HyperPAGE II Prestained Protein Marker) was used to estimate protein band size. Proteins were stained using Gelcode Blue Stain Reagent following the manufacturer's protocol (Thermo Scientific #24590; Rockford, IL). First the gel was washed three times in nanopure water at 5 minutes per wash. This step was included to remove residual SDS remaining on the gel. After washing, gels were stained with the Gelcode Blue Stain Reagent for one hour. After staining, gels were washed 3 more times using nanopure water at 30 minutes per wash. Gels were then held in a 10% Acetic acid solution until imaging or sequencing. Bands of interest were excised from gels and sent to the Purdue University Proteomics core facility for peptide sequencing on an ABI 4800 mass spectrometer. This mass spectrometer uses matrix assisted laser desorption ionization for sequencing peptides/proteins. Peptides were identified using the protein BLAST tool on NCBI. The translated transcriptome of the German cockroach was also used for protein identification (Gondhalekar et al. unpublished data).

Comparative expression of BGTG and Bla g 2 transcripts. For whole body gene expression experiments, adult males, non-gravid and gravid females were isolated from the rearing colony. Adult males and females aged for 5-7 days post imaginal molt were used. Five insects of each sex and life stage were dissected and placed in 250µl of RNA*later* stabilization solution. For individual body part experiments, body parts from 5 insects, (head, wing, alimentary canal, 7^{th} and 8^{th} tergites, the leftover carcass and for gravid females, the ootheca), were dissected and placed into separate 1.5 ml microcentrifuge tubes containing 250µl of RNA*later* stabilization solution. Whole body

insects and body parts immersed in RNA*later* were stored at 4°C or -20°C until RNA isolation

RNA isolation was done with the SV Total RNA Isolation System (Promega, Madison, WI). Three independent RNA isolations representing 3 biological replicates were performed for each whole body or body part preparation. Once isolated, RNA was held at -80^{o}C until used for cDNA synthesis. The RNA isolated from whole bodies and individual body parts was used to synthesize cDNA using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to manufacturer instructions. The complete reaction mixture of each cDNA reaction was incubated in a thermal cycler for 5 minutes at 25^{o}C, 30 minutes at 42^{o}C, and 5 minutes at 85^{o}C. Once synthesized, cDNA was held at -20^{o}C until use in quantitative real time PCR (qRT-PCR) experiments.

All qRT-PCR experiments were done on 96 well plates for the target genes BGTG and Bla g 2. Ribosomal protein L13a (RSP L13a) and pGEM were used as endogenous and exogenous reference genes for whole body treatments and body tissue tests, respectively. Three technical replicates were performed for each biological replicate. Each 20µl reaction mixture included 10µL SYBR® SensiMix (Bioline, Tauton, MA), 1µL each of forward and reverse primers (0.5 µM final concentration), 1µL cDNA and 7µL nanopure water. Primer sequences are shown in Table 1. The following thermal cycler program was used: an initial denaturing step at 95^{o}C for 10 min; 45 amplification cycles of 94^{o}C for 30 sec, 60^{o}C for 30 sec, and 72^{o}C for 30 sec; an extension step at 72^{o}C for 10 min; and a final melting curve step (90 cycles of temperature reduction from 90^{o}C to 50^{o}C at a rate of 0.5^{o}C/10 sec). The critical threshold (CT) data obtained was analyzed by using the 2-$^{\Delta\Delta CT}$ method. Statistical significance of BGTG-1 and Bla g 2 expression

between whole body treatments was determined using Mann-Whitney U pairwise comparisons ($P<0.05$). Expression data for BGTG-1 and Bla g 2 in body tissue treatments were also compared statistically using the pairwise Mann-Whitney U tests ($P<0.05$). Global Kruskal-Wallis tests ($P<0.05$) were used to determine statistical significance of gene expression differences between 7^{th} and 8^{th} male tergites (MT) and other tissues of either males, non-gravid females or gravid females. Both of these statistical tests are non-parametric. I used non-parametric tests because my data did not follow normal distribution and had unequal variance.

2.4 Results

2.4.1 Protein Separation and Identification

Four major protein bands were visualized on SDS-PAGE gels after Coomassie staining. The relative size of these proteins determined using the HyperPage III Prestained Standards was 94kDa, 63kDa, 45kDa and 23kDa (Figure 1). One of these proteins, the 63kDa protein, has been previously identified as an alpha-amylase protein (BGTG-1) by Saltzman et al. 2006. Through MALDI peptide sequencing the identity of the 63 kDa protein was reconfirmed as alpha-amylase (BGTG-1) and the 45kDa protein band was identified as the well-characterized allergen Bla g 2. The identity of other two proteins (94 and 23 kDa) could not be identified via peptide sequencing.

2.4.2 Whole Body Gene Expression Comparison

Figure 2 depicts the whole body expression of BGTG and Bla g 2 in adult males (AM), adult females (AF) and adult gravid females (AGF). Using a Mann-Whitney U pairwise comparison, there was no significant difference ($p>0.05$) between the 3 groups for the BGTG-1 gene. For Bla g 2, there was a significant difference ($p<.05$) between the AGF and the AF groups. There was no significant difference ($p>.05$) between the AM group when compared to the AF and the AGF groups for the Bla g 2 gene.

2.4.3 Individual Tissue Gene Expression Comparison

BGTG-1 expression in different body tissues of adult males is portrayed in Figure 3. Male heads, (MH), male wings, (MW), male guts, (MG), the 5^{th} 6^{th} tergites, (5^{th} 6^{th}) and male carcasses, (MC), were all tested in the individual body part comparisons. The BGTG-1 gene showed significantly higher expression in MT (tergal glands) as compared to the head (MH), wings (MW), and carcass (MC) based on pairwise Mann-Whitney

comparisons ($P<0.05$). Global Kruskal-Wallis comparison of BGTG-1 expression in all body tissues except the tergal glands (MT group) did not show significant differences between tissues ($P>0.05$). Figure 4 depicts tissue gene expression comparisons for Bla g 2 in different tissues of adult males. All tissues (heads, wings, 5^{th} and 6^{th} tergites and carcass) showed significantly lower expression of the Bla g 2 gene in comparison to tergal tissue (MT). Again, the global analysis of all the groups minus the MT group showed no statistically significant differences in Bla g 2 expression.

For non-gravid adult females (AF), female heads, (FH), female wings, (FW), female guts, (FG), female tergites, (FT) and female carcasses, (FC) showed lower expression of BGTG as compared to male tergal glands (MT) (Figure 5). Every group was statistically different than the MT group when compared using pairwise Mann-Whitney comparisons. Using the Kruskal-Wallis global analysis also revealed significant BGTG expression differences between different body tissues of adult females. In Figure 6, expression for the same body tissues is compared for the Bla g 2 gene. The Bla g 2 gene was highly expressed in MT as compared to the FH, FW and FG groups. Comparison of all female body tissue groups minus the MT group also revealed significant differences in expression of Bla g 2.

Differences in expression of the BGTG-1 gene in gravid female heads, (GH), gravid female wings, (GW), gravid female guts, (GG), gravid female carcasses, (GC) and gravid female oothecae, (Ootheca) and male tergal glands (MT) are portrayed in Figure 7. In a pair-wise comparison, every body tissue of gravid adult females showed significantly lower expression of the BGTG gene as compared to the MT group. Using a global comparison, all the groups are different when all compared together minus the MT group.

In figure 8, the same groups compared in Figure 7 are again compared for Bla g 2 expression. The GH, GG, GC and ootheca groups are all statistically different than the MT group when compared in pair-wise comparisons. In the global analysis, there was no significant difference in Bla g 2 expression between different body tissues of gravid females.

2.5 Discussion

2.5.1 Protein Separation and Identification

This study revealed 4 major proteins of different sizes (94, 63, 45 and 23 kDa) after separation by SDS-PAGE gel (Figure 1). Similar results were also observed in a previous study by Saltzmann et al. (2006). In the study by Saltzmann et al. (2006) the 63 kDa protein was identified as an alpha-amylase enzyme and named “BGTG-1”. The alpha-amylase protein is unique to the tergal glands and was not detected in other cuticular protein samples. Using peptide sequencing and subsequent protein BLAST search in Genbank the 45kDa protein was identified as Bla g 2. Since Bla g 2 is a ubiquitous allergen protein found on the body and throughout the environment inhabited by the German cockroach (Arruda et al. 1995, Pomés et al. 2002), one possibility is that the Bla g 2 protein produced elsewhere in the body was contaminating the tergal secretion. Previous reports on Bla g 2 expression indicated that it is predominantly found in the cockroach midgut (Arruda et al. 1995). Bla g 2 is an important allergen protein, as it is reported that in some cities and towns upwards of 60% of asthma patients are allergic to cockroach allergens such as Bla g 2 (Arruda et al. 1995). Given the importance of Bla g 2 as an allergen and the surprising finding of Bla g 2 expression in the male tergal glands, I further verified the expression of Bla g 2 using qPCR, not only at the whole insect level but also in individual cockroach body parts including male tergal glands (MT). The tergal gland-specific alpha amylase gene served as a positive control in these qPCR experiments.

2.5.2 Whole Body and Tissue-Specific Gene Expression Comparison

When looking at BGTG and Bla g 2 expressions in whole bodies of insects the results are quite different in that there is no difference in BGTG expression between whole body samples, but there is a difference seen between the AGF and AF groups in Bla g 2 expression. In the whole body comparison experiment, though, it should be noted that this could be due to the fact that these two sets of genes are being diluted in the sample. This led me to go into the direction of looking at tissue-specific expression of these two genes to get a better picture as to where these genes are being expressed and where the highest levels of expression are seen. This information can lead to an understanding of where these genes are found throughout the cockroach body.

2.5.3 Individual Tissue Gene Expression Comparison

BGTG-1 and Bla g 2 are two specific proteins found in the German cockroach (Saltzmann et al. 2006, Gore and Schal 2007). BGTG-1 is a 63 kDa alpha-amylase protein found specifically in the tergal glands of adult male German cockroaches, where its proposed function is the freeing of phagostimulatory sugars from longer chain oligosaccharides (Saltzmann et al. 2006, Nojima et al. 1999). Bla g 2 is a ~36-45kDa protein that shows sequence homology to aspartic proteases. Due to its high concentrations being found in the digestive organs of the German cockroach, Bla g 2 is proposed to be an important digestive enzyme (Arruda et al. 1995, Pomés et al. 2002). Based on qPCR experiments, the BGTG-1 gene is only significantly highly expressed when compared to the MH, MW and MC groups, which might suggest that in the MG and 5^{th} 6^{th} tergite groups there was some contamination during dissections from either the tergal glands or alimentary canal (Figure 3). In this

regard, it is likely that the digestive (gut) alpha-amylases could also be amplified by the BGTG qPCR primers. In contrast, Bla g 2 relative expression is highest in the male tergal glands (MT) when compared to all other body parts of adult males (Figures 4). These results for Bla g 2 confirm that this allergen gene is expressed in the tergal glands of adult male cockroaches and thus provides strong evidence in support of the finding that Bla g 2 is one of the major proteins constitutively produced in the tergal gland secretion. Another interesting finding is that BGTG and Bla g 2 expression in the male tergal glands was higher compared to most of the adult female and gravid female body parts (Figures 5–8). These results were expected for BGTG-1 since it is a tergal gland specific protein. However, this is a surprising finding for Bla g 2 since it is a ubiquitous protein thought to be highly expressed in the cockroach gut and found in high concentrations in their frass when excreted into the environment (Arruda et al. 1995). One explanation for the high expression of Bla g 2 in the male tergal glands is that Bla g 2 might be serving the function of a ligand-binding protein, where it might be binding small hydrophobic ligands such as hormones (Wünschmann et al. 2005).

The finding that Bla g 2 is highly expressed in the male tergal glands is a major outcome of my work and warrants further studies for identifying the functional role of Bla g 2 in tergal glands. However, because the BGTG-1 (alpha-amylase) is a well-defined tergal gland specific protein, I decided to look at the BGTG-1 protein in more detail and see its potential effectiveness as an alpha-amylase enzyme that converts long chain sugars into simple phagostimulatory sugars, like maltose, found in the tergal glands of male German cockroaches (Nojima et al. 1999, 2002).

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CHAPTER 3. GERMAN COCKROACH TERGAL GLAND ALPHA-AMYLASE ACTIVITY

3.1 Abstract

BGTG-1 is a tergal gland specific alpha-amylase protein thought to hydrolyze long chain oligosaccharides to produce maltose. In these experiments, I performed different tests to investigate how well BGTG-1 could produce or release maltose from starch. RNA interference (RNAi) was performed to silence BGTG-1 gene expression by injecting BGTG-homologous double-stranded RNA (dsRNA) into male cockroaches. When comparing RNAi silencing efficiency groups injected with BGTG-1 dsRNA showed significantly lower BGTG-1 expression as compared to pGEM injected control groups, and this correlated with results of starch hydrolysis assays showing significantly reduced maltose release in association with BGTG-1 knockdown. To see if other sugars were contributing and creating background noise during the maltose assay, I performed a glucose detection assay and found that the BGTG-1 and pGEM injected groups were not significantly different, which implies that at least glucose interference is not an issue. These results show clearly that BGTG-1 RNAi has an effect on the amount of maltose production in tergal gland tissues. This is important because maltose is the major stimulatory factor attracting females to feed from the tergal glands, leading eventually to mating between a male and female. These findings also provide a framework for future studies on maltose

production in tergal glands *in vivo* and the specific role of maltose in cockroach mating behavior and biology.

3.2 Introduction

German cockroach tergal glands are specialized epithelial cells that secrete a mixture of different compounds for the primary purpose of offering this substance to a female in an attempt to mate (Saltzmann et al. 2006b). The different types of epithelial cells that make up the tergal glands include: epidermal cells, associated cells that have microtubules within them, and specialized secretory cells (Saltzmann et al. 2006a). Although the secretion is used in mating to attract females, it is not considered sex-specific, but as a dietary feeding stimulant because nymphs and other male cockroaches that are close by will feed on the tergal gland secretion as well (Nojima et al. 1999).

Four main proteins have been isolated and separated on a SDS-PAGE gel from the German cockroach secretion. These proteins are approximately 23, 45, 63 and 94kDa in size (Saltzmann et al. 2006a and Chapter 2). Of particular interest is the 63kDa protein because this is specific to the tergal gland secretion. This protein has been identified as an alpha-amylase protein and named BGTG-1 (Saltzmann et al. 2006b). Alpha-amylase proteins serve the function of hydrolyzing α(1-4) glycosidic linkages in large chain sugars, such as starch (Maarel et al. 2002). To date, only one other alpha-amylase is known to exist outside of the digestive tract and it is found in the hypopharyngeal glands of honeybees (Ohashi et al. 1999).

In addition to proteins, the main component found in the tergal glands of male German cockroaches is a complex oligosaccharide mixture. These oligosaccharides, which include but are not limited to maltose, maltotriose and maltotetraose (Nojima et al. 1999) are thought to serve the primary function of attracting the female to feed

from the tergal gland secretion (Nojima et al. 2002). A possible and proposed function of BGTG-1 is that it is hydrolyzing longer chain polypeptides to liberate the phagostimulatory oligosaccharide fraction of the tergal gland secretion (Saltzmann et al. 2006b). In the study reported below, my objective was to determine if BGTG-1 is serving this function by performing BGTG-1 RNAi in combination with enzyme assays, using starch as the substrate for the enzyme.

3.3 Materials and Methods

Double stranded RNAs (dsRNAs) used for the RNAi injections in this experiment were synthesized using the MEGAscript® RNAi kit following the manufacturer's instructions. Primer sequences used for making dsRNA can be found in Table 2. Male 5th instar nymphs were isolated and a grand total of 90 insects were injected with 1µg of either BGTG or pGEM dsRNA or nuclease-free water (30 insects with BGTG dsRNA, 30 insects with pGEM dsRNA and 30 insects with nuclease-free water). pGEM is a bacterial plasmid DNA sequence (www.promega.com/vectors/) that should not be homologous to any cockroach sequences. Thus, the pGEM dsRNA served as an exogenous dsRNA control (i.e., it was used to verify that observed RNAi effects were BGTG dsRNA-specific and not simply caused by injecting foreign dsRNA into cockroaches). The number of insects injected per treatment allowed for 3 biological reps per treatment. Once injected, insects were allowed to molt to their adult form and were aged 7 days before tergal glands were dissected out. Protein isolations and protein concentration determinations followed the same protocol as given in Chapter 1.

Starch hydrolysis assays were as follows. On a 96 well plate, maltose standards ranging from 0.025 to 4mg/ml were pipetted into 3 wells per standard respectively. For standards, each well received 20µl of maltose and 30µl of sodium phosphate buffer (pH 7.0). For test wells, which were also done in triplicate to give 3 technical reps per biological rep, each well received 15µl of the enzyme or protein from the isolations and 35µl of starch solution (1mg/mL). Samples were allowed to incubate for 1 hour at 30°C. Following incubation, test plates were submerged in a

boiling water bath for 5 minutes, then 150µl of DNSA solution was added to all the wells to stop the reaction. DNSA solution consisted of 1% 3, 5- DNSA, 30% potassium tartarate and 0.4M NaOH. Plates were then allowed to dry for a few minutes before being loaded on a plate reader and being read at 540nm. The resulting absorbance data were transferred to Microsoft Excel for data analysis. Statistical comparisons of mean activity data were done using Mann-Whitney U pairwise comparisons between two groups.

Preliminary DNSA enzyme assay experiments were performed to standardize pH of assay buffer, insect age, incubation time and isolation method for tergal secretion. The standardization experiments were repeated for 3 technical reps the same as mentioned above, but only for one biological replicate. Because the DNSA reagent also detects other sugars, glucose detection assay were also run to investigate for possible interference from other sugars. For the glucose assay, the plate set up was the same except instead of maltose standards, glucose was used. A glucose detection kit was used and the protocol provided by the manufacturers was followed (Glucose Detection Kit; Wako Chemicals USA, Richmond, VA) (Scharf et al. 2011). The data and statistics were computed in the same fashion as in the maltose assay.

3.4 Results

Extensive preliminary studies were conducted to establish that dsRNA injections as performed result in (i) over 90% BGTG-1 transcript silencing and (ii) a similar level of protein attenuation for BGTG-1 RNAi treatments relative to pGEM controls (A.D. Gondhalekar, unpublished results). When looking at Figure 9, there was a significant difference ($p<.05$) in maltose detection between the BGTG and pGEM injected groups. The H_2O injected group was not significantly different ($p>.05$) in maltose release than either the BGTG-1 or the pGEM injected groups. In Figure 10, BGTG-1 was not significantly different in glucose release than either the H_2O or the pGEM injected groups. There was a significant difference in glucose detection between the H_2O and pGEM injected groups in Figure 10, but the results do not seem to be BGTG-1 RNAi-specific.

3.5 Discussion

In Figure 9, we were able to see that the BGTG-1 dsRNA injected group showed significantly less maltose release than the pGEM group. This is an interesting result because the pGEM group here is the true control group showing that just injecting foreign dsRNA into the cockroaches is not having an effect on the ability of BGTG-1 to release maltose from starch, but rather, that injecting dsRNA specifically corresponding to BGTG-1 is reducing activity. These results was also verified at the mRNA and protein levels through extensive preliminary studies (A.D. Gondhalekar, unpublished results). However, since using the DNSA detection method can potentially provide unclear results (because DNSA will measure any reducing sugars), looking at Figure 10 with the glucose detection assay strengthens our result for Figure 9 because there is no significant difference between BGTG-1 and pGEM injected groups. This shows that both these groups are being affected in a similar fashion by apparent "background noise" contributed by glucose. Therefore, my findings here provide a clear link between the BGTG-1 gene and α-amylase activity in the tergal gland.

This function of BGTG-1 is not unique to German cockroaches. In male tergal glands of the Madeira cockroach, *Leucophaea maderae*, there is a β-glycosidase enzyme, named Lma-p72. This enzyme has two proposed functions: firstly, it could hydrolyze native oligosaccharides, thus stimulating female feeding and secondly, it could cleave a pheromone-sugar conjugate to release a pheromonal chemical onto the body surface (Saltzmann et al. 2006b, Cornette et al. 2003). These two very different enzymes found in the tergal glands of different cockroach species could be

performing a similar task in hydrolyzing oligosaccharides, and thus releasing phagostimulatory sugars in an attempt to attract a female to feed from the tergal gland secretion.

The release of maltose by this protein is important because maltose has been shown to be one of the most powerful feeding stimulants for German cockroaches (Nojima et al. 1996, Tsuji 1965). The presence of maltose in high amounts and the presence of longer chain oligosaccharides in the tergal glands of male cockroaches suggests that a protein or some other factor is cleaving these longer sugars to create the stimulatory compound, maltose. Knocking down this protein through RNAi as was done here could possibly affect German cockroach mating behavior. For example, with this protein being knocked down there is nothing to free maltose from the oligosaccharides present. It is therefore possible that female cockroaches would not be attracted to BGTG-silenced males when they lifted their wings to expose their tergal glands prior to mating. However, preliminary research has shown that knocking down this protein has no effect on mating success or on the number of progeny produced within the ootheca of the female (K. Pluchar & A.D. Gondhalekar, unpublished results).

Despite these uncertainties relating to behavioral impacts, my findings clearly link the BGTG-1 gene and protein to α-amylase activity in the cockroach tergal gland. This work also provides the first example in the German cockroach of a connection between a gene and enzyme activity through the integrated use of RNAi and enzyme biochemistry. Future research should compare the effects of BGTG-1 silencing on the sugar composition within the tergal glands of knockdown and non-knockdown insect.

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CHAPTER 4. CONCLUSIONS

Two proteins, BGTG-1 and Bla g 2, were identified from the SDS-PAGE gels. These two protein's transcripts showed the highest expression in the tergal glands of the male German cockroach in every comparison (although it was not statistically significant in every comparison). This is important for several reasons: First, BGTG-1 has been identified and found in past research to be specific to the tergal glands of male German cockroaches. My research here confirms this work by showing the protein being separated on an SDS-PAGE gel and by showing the transcript of BGTG-1 having the highest relative expression in the tergal glands. Secondly, Bla g 2 is showing the highest relative expression in the tergal glands. Bla g 2 is an important cockroach allergen and it is known to be found in the gut and the frass of German cockroaches. Future research can be done to see if there is any transfer of this protein from the tergal glands to the gut and vice versa and to try to determine the function of this protein, especially as it relates to the tergal gland secretion.

Another important conclusion from my research is that I was able to knockdown BGTG-1 using RNAi and knocking down this protein has shown reduced release of reducing sugars (since DNSA is a non-specific assay method, I could not assume that it is reduced maltose release). Future work in this area would be to use specific assays for maltose release to verify this result and to use mass spectrometry to see differences in the

tergal gland secretion between knockdown and control insects. What's important about my findings here is that this is the first example in cockroaches that RNAi was used to determine the function of a protein.

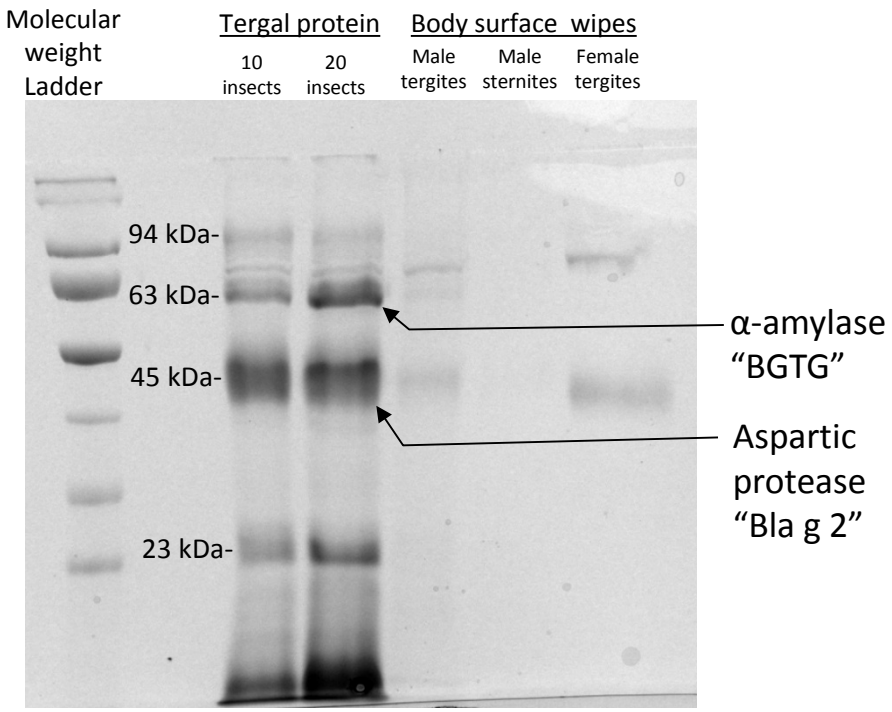

Figure 1 SDS-PAGE gel depicting 4 proteins of interest marked with their respective size next to them, with the 63kDA and 45kDa protein being identified as BGTG and Bla g 2, respectively.

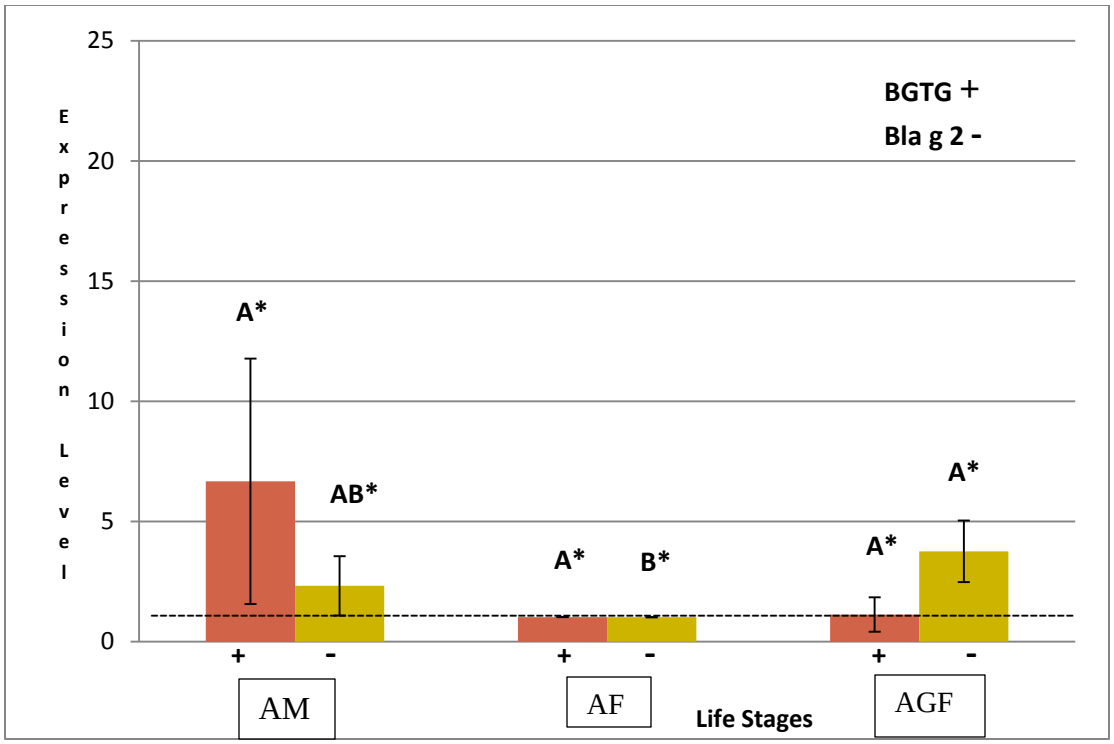

Figure 2 BGTG and Bla g 2 expression in whole body comparisons of adult males (AM), adult females (AF) and adult gravid females (AGF) *Blattella germanica*. BGTG bars are marked with a + sign and Bla g 2 bars are marked with a – sign. All the life stages were normalized to the AF group and statistical analyses were done using the delta-delta-CT values. Statistics were run using the Mann-Whitney U pair wise comparison. The dotted line represents the normalizing values.

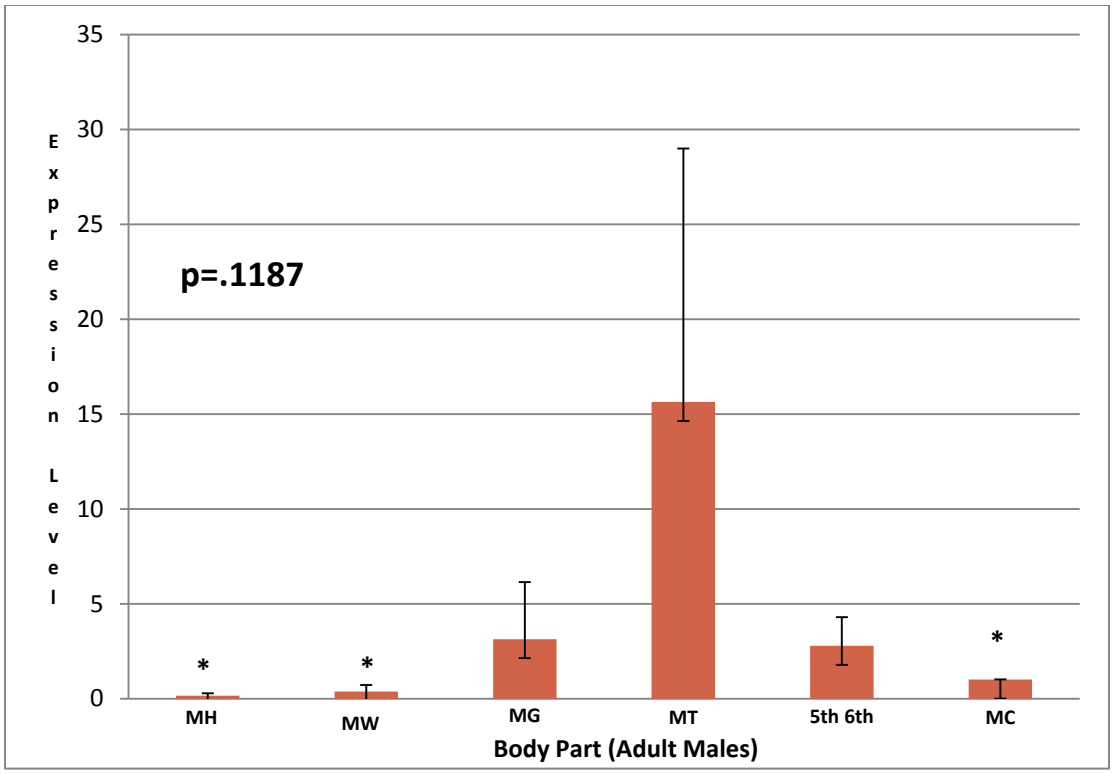

Figure 3 BGTG expression in male heads (MH), male wings (MW), male guts (MG), male tergal glands (MT), male 5^{th} 6^{th} tergites (5^{th} 6^{th}) and male carcasses (MC). All the life stages were normalized to the MC group and statistical analyses were done using the delta-delta-CT values. Statistics were run using the Mann-Whitney U pair wise comparison and asterisks represent statistically significant difference with the MT group. The p value represents a Kruskal-Wallis global comparison of all the groups minus the MT group.

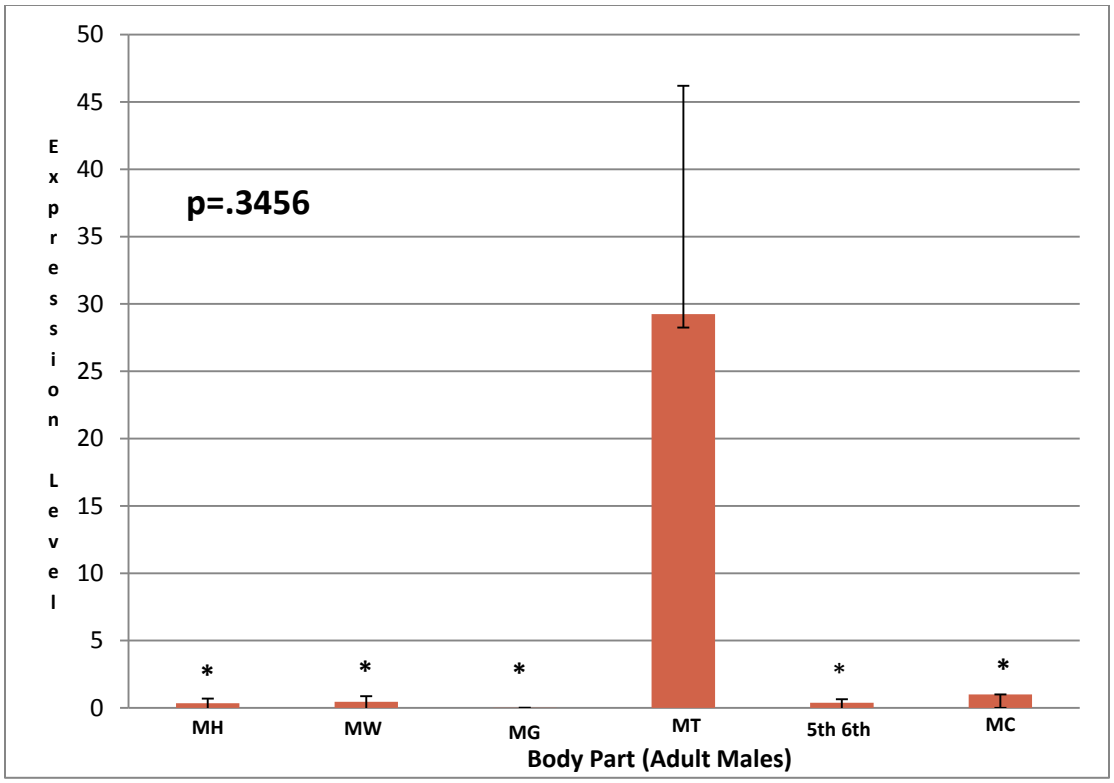

Figure 4 Bla g 2 expression in male heads (MH), male wings (MW), male guts (MG), male tergal glands (MT), male 5^{th} 6^{th} tergites (5^{th} 6^{th}) and male carcasses (MC). All the life stages were normalized to the MC group and statistical analyses were done using the delta-delta-CT values. Statistics were run using the Mann-Whitney U pair wise comparison and asterisks represent statistically significant difference with the MT group. The p value represents a Kruskal-Wallis global comparison of all the groups minus the MT group.

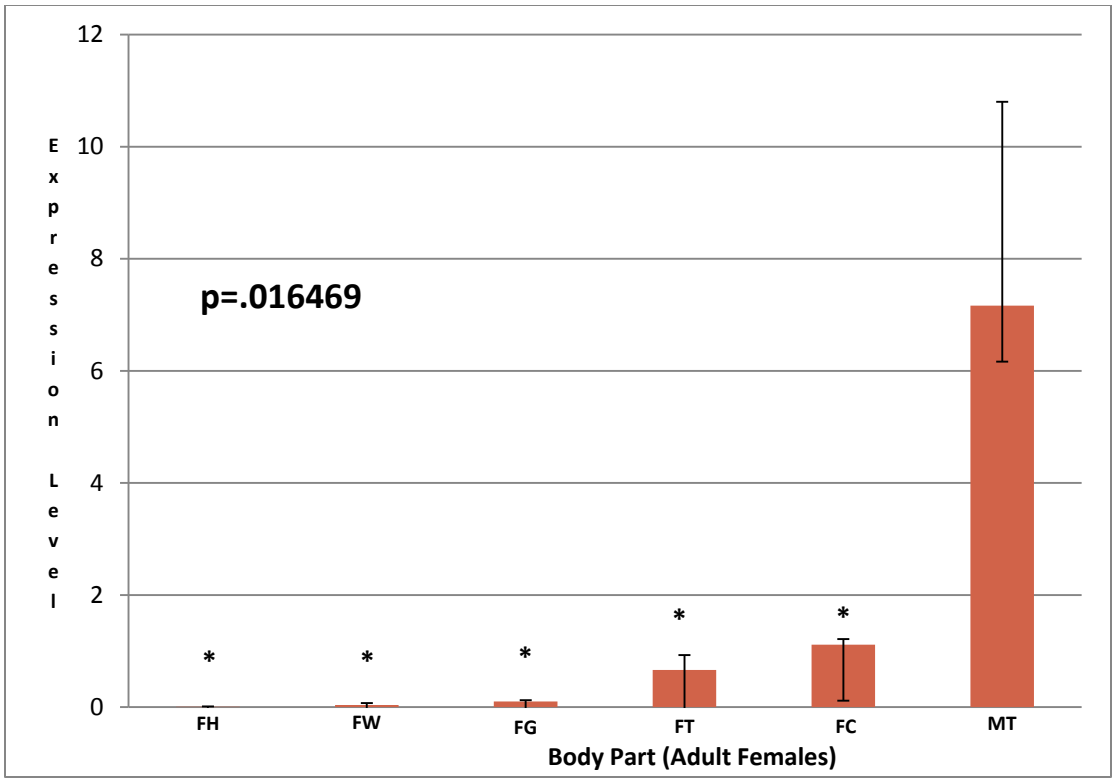

Figure 5 BGTG expression in female heads (FH), female wings (FW), female guts (FG), female tergites (FT), female carcasses (FC) and male tergal glands (MT). All the life stages were normalized to the FC group and statistical analyses were done using the delta-delta-CT values. Statistics were run using the Mann-Whitney U pair wise comparison and asterisks represent statistically significant difference with the MT group. The p value represents a Kruskal-Wallis global comparison of all the groups minus the MT group.

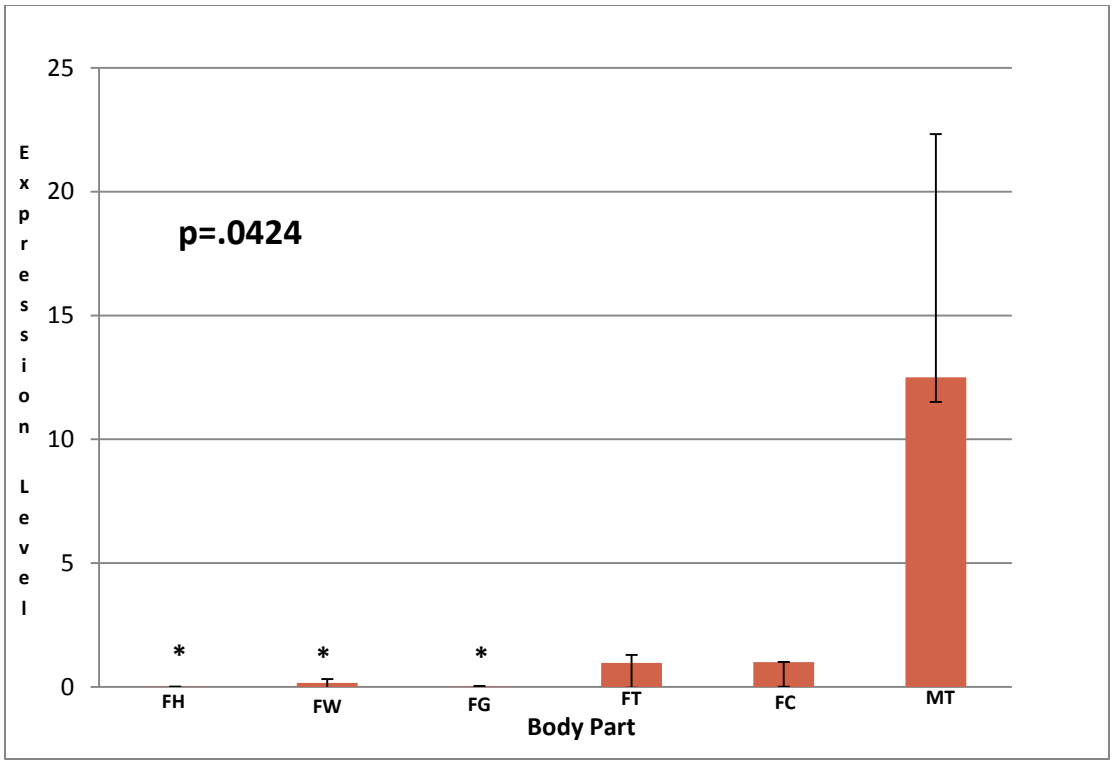

Figure 6 Bla g 2 expression in female heads (FH), female wings (FW), female guts (FG), female tergites (FT), female carcasses (FC) and male tergal glands (MT). All the life stages were normalized to the FC group and statistical analyses were done using the delta-delta-CT values. Statistics were run using the Mann-Whitney U pair wise comparison and asterisks represent statistically significant difference with the MT group. The p value represents a Kruskal-Wallis global comparison of all the groups minus the MT group.

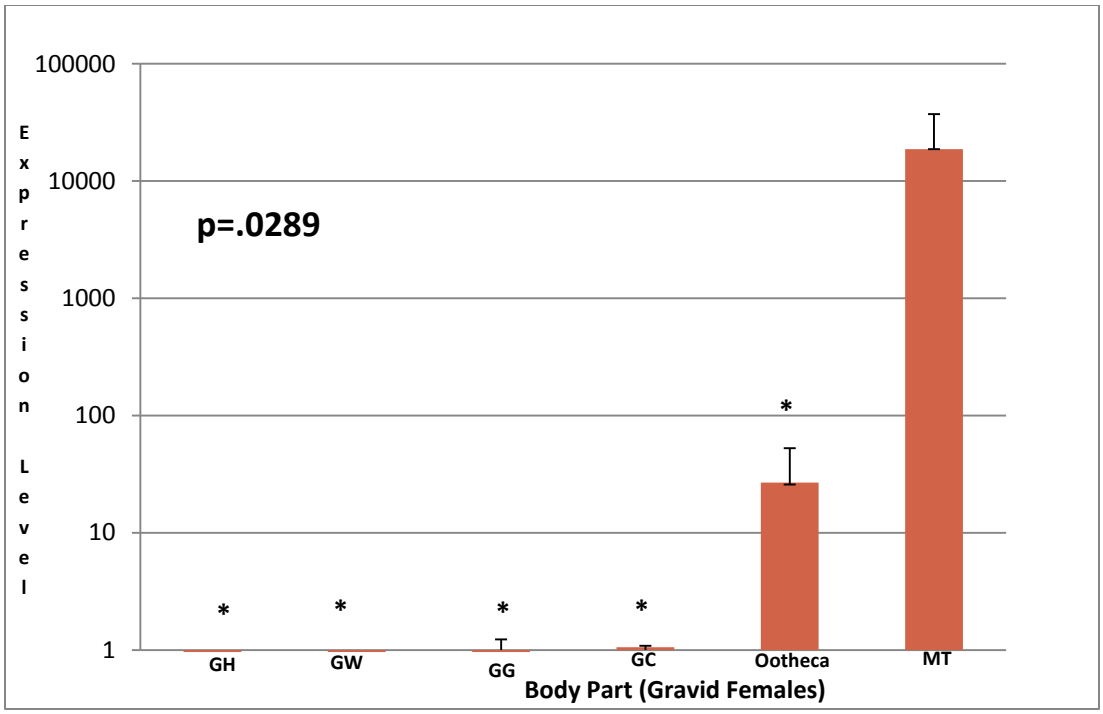

Figure 7 BGTG expression in gravid female heads (GH), gravid female wings (GW), gravid female guts (GG), gravid female carcasses (GC), oothecas (ootheca) and male tergal glands (MT). All the life stages were normalized to the GC group and statistical analyses were done using the delta-delta-CT values. Statistics were run using the Mann-Whitney U pair wise comparison and asterisks represent statistically significant difference with the MT group. The p value represents a Kruskal-Wallis global comparison of all the groups minus the MT group.

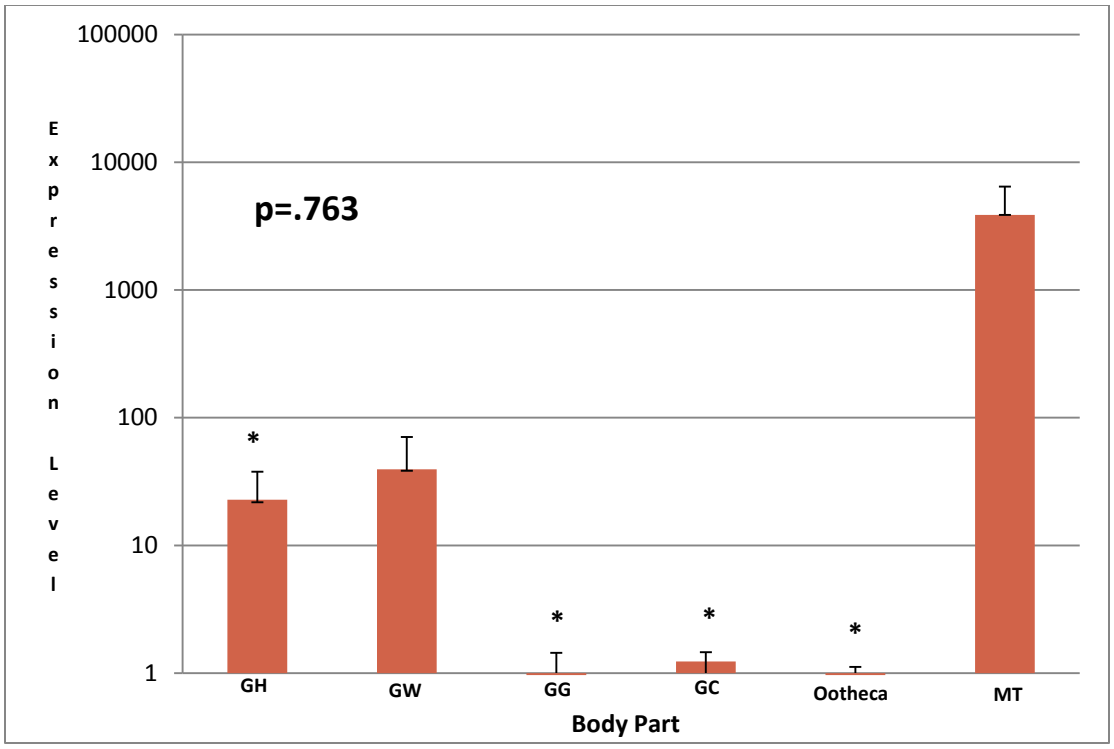

Figure 8 Bla g 2 expression in gravid female heads (GH), gravid female wings (GW), gravid female guts (GG), gravid female carcasses (GC), oothecas (ootheca) and male tergal glands (MT). All the life stages were normalized to the GC group and statistical analyses were done using the delta-delta-CT values. Statistics were run using the Mann-Whitney U pair wise comparison and asterisks represent statistically significant difference with the MT group. The p value represents a Kruskal-Wallis global comparison of all the groups minus the MT group.

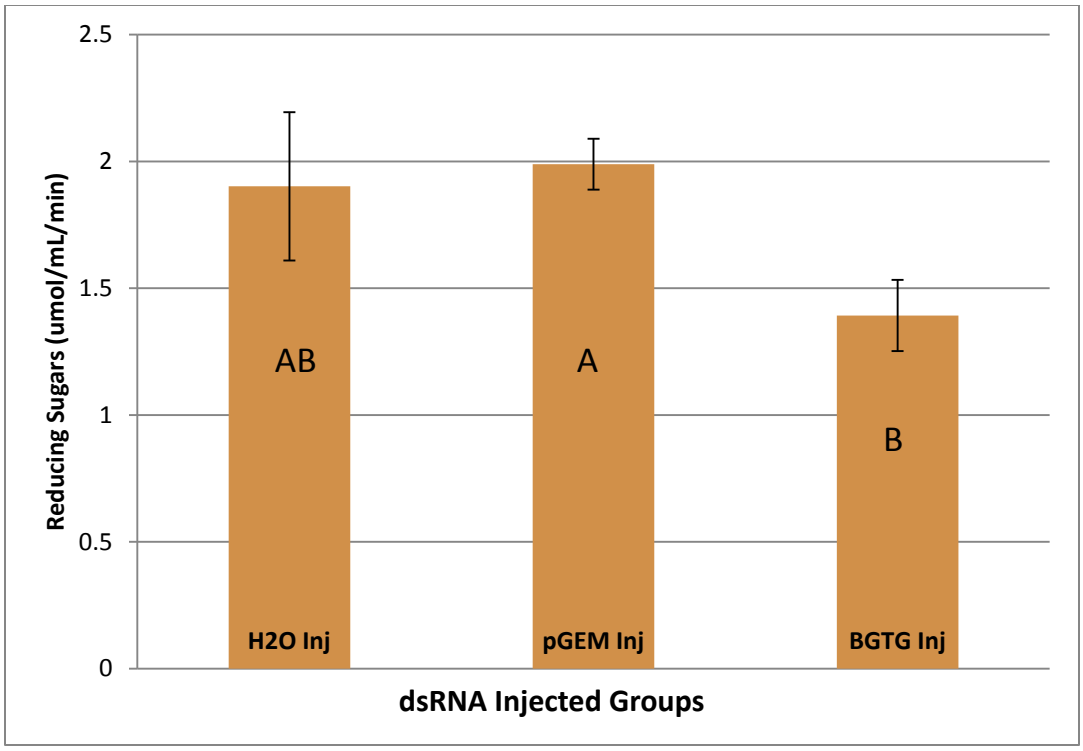

Figure 9 Reducing sugars in dsRNA injected groups (all adult male insects). Statistical analyses were run using the Mann-Whitney U pair wise comparison. A and B represent a statistically significant difference between groups. AB represents a difference, but not a statistically significant difference to either the A or B analysis.

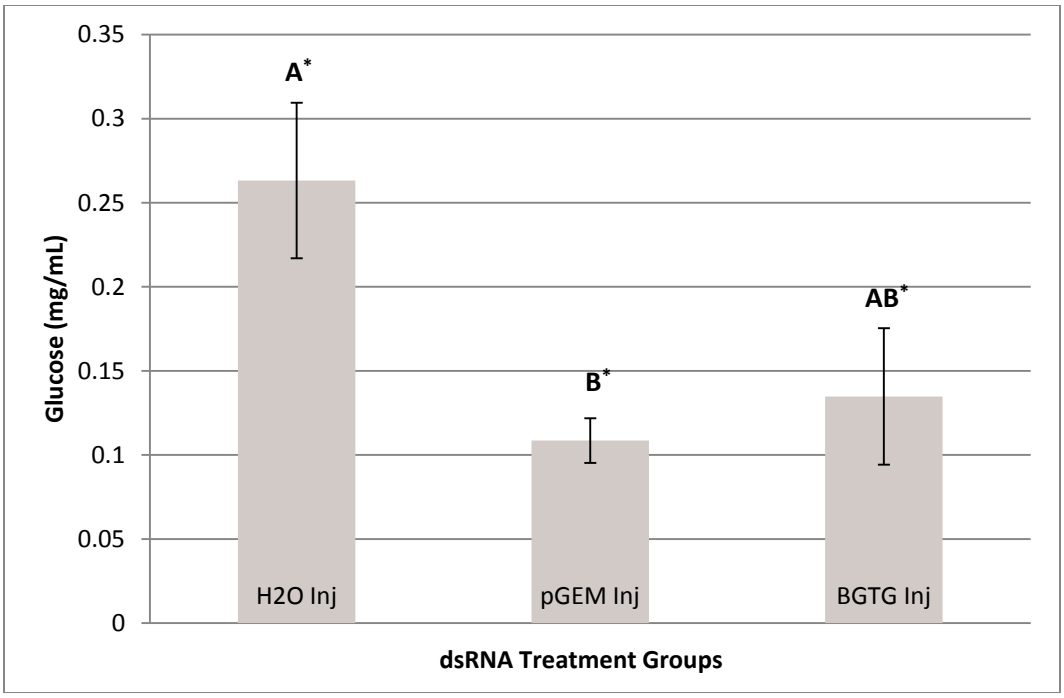

Figure 10 Glucose detection in dsRNA treatment groups (all adult male insects). Statistical analyses were run using the Mann-Whitney U pair wise comparison. A and B represent a statistically significant difference between groups. AB represents a difference, but not a statistically significant difference to either the A or B analysis.

Table 1 **:** Primers used for qPCR experiments

Gene/ Primer ID	**Forward primer (5’ to 3’)**	**Reverse primer (5’ to 3’)**
BGTG	CTGAGTTTGGACGGGTGACT	GGGCAAAGTGTGTGTGAGAA
Bla g 2	GCCCTAATGCACTGAAAGGA	TCCAATCAGAACCTCCGAAA
Ribosomal Protein L13a (RSPL13a) – reference gene	AGACCCAAGTCACGCAAGAT	TGGTTTGGCAACAACATCAG
M13 (pGEM reference gene primers)	GTAAAACGACGGCCAGT	AACAGCTATGACCATG

Table 2 : Primers used for dsRNA synthesis

Gene/ Primer ID	**Forward primer (5’ to 3’) with T7 recognition sequence appended on 5’ end**	**Reverse primer (5’ to 3’) with T7 recognition sequence appended on 5’ end**
BGTG	TAATACGACTCACTATAGGG-TGTCGACAGCCTACTCTATGTACG	TAATACGACTCACTATAGGG-TGCCACTAGGGCAAAGCATACTGA
PGEM (exogenous dsRNA control)	TAATACGACTCACTATAGGG-GGTATCAGCTCACTCAAAGG	TAATACGACTCACTATAGGG-GAACGACCTACACCGAACT